PRODUCTION, ISOLATION, IMMOBILIZATION AND APPLICATION OF GLUCOAMYLASE FROM RHIZOPUS ORYZAE

A.T. Karossi and L.Z. Udin

Research Center for Chemistry – LIPI
Jalan Sangkuriang – Bandung 40135
Indonesia
(E-mail : andi012@lipi.go.id or karossi@ bdg.centrin.net.id)

ABSTRACT

Experiments were conducted to find optimum medium and process conditions based on shake-flask and laboratory fermentor scales to anticipate industrial scale for the production of glucoamylase. Membrane filtration and ammonium sulfate precipitation were tested for the enzyme concentration and isolation respectively. The glucoamylase enzyme properties were determined and preference to various starch substrates were checked. Immobilization within commercial agar and alginate were done and their shelf-life were observed. The application of free glucoamylase in saccharification of the various starches were also investigated after being liquefied with commercial alpha-amylase.

Keywords : rhizopus oryzae, glucoamylase, isolation, immobilization.

INTISARI

Beberapa percobaan dilakukan untuk mendapatkan media optimum dan kondisi yang didasrkan pada skala labu-kocok dan fermentor maboratorium, dalam mempersiapkan skala industri dari produksi gulokoamilase. Filtrasi membrane dan pengendapan dengan ammonium sulfat diujikan masing-masing untuk pemekatan dan isolasi enzim. Sifat-sifat enzim glukoamilase ditentukan dan prefernsinya terhadap berbagai substrat pati diperiksa. Amobilisasi pada agar komersial dan alginate dilakukan dan waktu tahan-simpan diamati. Penggunaan glukoamilase bebas pada proses sakarifikasi dari berbagai pati di atas setelah dicairkan dengan alfa amylase komersial, juga diselidiki.

Kata Kunci : rhizopus oryzae, glucoamylase, isolation, immobilization.

INTRODUCTION

Properties and applications of starch-converting enzymes of the alpha-family have been reviewed thoroughly elsewhere. Glucoamylase (E.C. 3.2.1.3) belongs to the exoamylase which cleaves both alpha,1-4 and alpha,1-6 glycosidic bonds. It acts on the external glucose residues of amylose or amylopectin and thus produces only glucose. Application for starch saccharification employs amyloglucosidase or glucoamylase besides pullulanase, maltogenic alpha-amylase, alpha amylase and isoamylase (v.d.Maarel et.al, 2002). On the other hand the research and development status of the starch converting enzymes in Indonesia up to the latest 1980 decades including the production of glucoamylase had been listed (Karossi et al, 1990, 2005). Fermentation media used in producing the enzyme mostly of semi-synthetic and were locally produced together with Aspergillus or Rhizopus as fermenting microorganisms. Yearly import of glucose to Indonesia significantly increased and conversely, investment on glucose making industries tend to decrease by reason of higher cost and unavailability of tapioca starch, which is used as raw material in the industry.. Sago (Metroxylon) starch is abundantly available in the country and has great potential to replace tapioca starch.

The present paper describes progress achieved on glucoamylase research conducted in our laboratory on the production, isolation and application of Rhizopus oryzae glucoamylase.

EXPERIMENTS AND RESULTS

1. Production of the glucoamyl

1.1. Production in shake flask experiments

Initially set of experiments on the production of the glucoamylase was conducted in Erlenmeyer flasks using medium containing sago (Metroxylon sp.) starch as carbon source and soybean meal as nitrogen source as a simple medium and found that 2% of sago starch and 0.5% of soybean meal in the medium was the best medium composition for the production of the glucoamylase. Further experiments were carried out in a fermentation medium using sago starch with various levels of mung bean sprout extract (1 - 5 %) added into the medium as vitamin source. The experiment was carried out at 30° C for five days in aerobic condition with agitation rate of 120 rpm. For comparison another fermentation medium containing malt extract 3% instead was also tested. The glucoamylase specific activity of 4500 U/g protein, starch consumption of 62.5% and biomass produced of 2.93 g dry weight/ L medium were demonstrated with the latter medium. It was found that media containing 4% mung bean sprout extract had maximum glucoamylase specific activity of 19720 U/g protein at day-3. The enzyme activity was assayed at 55° C with incubation time 10 minutes. At this third day of fermentation the starch utilization reached 55.5% and the biomass production was 3.12 g dry weight / L medium (Iskandar et al, 1994; Udin et al, 1994). It is clear that for the shake flask scale with 50 ml medium volume the 4% mung bean sprout extract was superior to malt extract. Nevertheless for larger scale fermentation the use of mung bean extract may not be appropriate.

1.2. Production in laboratory fermentors

Further experiments to disclose the optimum condition for maximum production of the glucoamylase was investigated using sago starch medium in 2L and 4L scale laboratory fermentors (Udin et al, 1994). The results showed that the maximum production of the glucoamylase at 600 - 1500 ml fermentation scale was reached at day-8 of incubation time. At this condition, the enzyme specific activity was 850 - 1500 U/ g protein. For the glucoamylase production within 4L fermentation scale, the maximum specific activity , 2580 U/g protein was obtained at day-8 with 300 rpm agitation while the maximum activity of 3470 U/g protein and 4710 U/g protein were achieved at day-6 and day-5 of fermentation process with 500 rpm and 700 rpm agitation, respectively. At this maximum condition , the use of sago starch reached 94 - 98 %, and biomass production at the end of fermentation process was 4.80 - 7.90 g dry weight / L medium.

An intention for scaling up the conditions to 100 L capacity fermentor dictated a further experiment using 10 L scale laboratory fermentor (Siagian et al, 1994; Surawidjaja et al, 1996). The glucoamylase was then produced in a 10L stirred tank fermentor using sago starch as the main medium component. The fermentation condition were adapted from the results obtained from shake flask and the 4L fermentor experiments. The temperature was set at 30° C, pHs were at a constant value of 4.0, 4.5, 5.0, 5.5 and 6.0. Agitation rate at 1.5 vvm were adjusted at 286 rpm, 300 rpm, and 350 rpm with medium volume of 6 L. The maximum production condition of glucoamylase was reached as listed in Table 1.

Table 1. Fermentation process condition for maximum production of glucoamylase.

Agitation rate	350 rpm
Aeration	1.5 vvm
pH	4.0
Fermentation time	5 days
Specific activity	13631 U/g protein.
Average kLa	43 /hour.

The possibility of having a higer enzyme productivity when maltose as inducer was added within the first three days of fermentation, have been reported and an empirical formula was build (Surawidjaja et al, 1996) to correlate the concentration of maltose added and the day of fermentation being introduced. The regression is:

ESA = 7553.135 + 7844.165 * day - 9939.290*(day/[maltose])(r^2=0.93) ; ESA=Enzyme Specific Activity

2. Concentration and Isolation of the glucoamylase

2.1. Membrane filtration

Preparation of the glucoamylase was by fermentation of sago starch and soybean meal in the laboratory fermentor scale. Enzyme concentration was carried out by ultra-filtration process, where polysulfone membranes were used as medium filter. Membranes used in these experiments were prepared with several treatments (Syahril et al,1993). The best membrane for the enzyme concentration by ultrafiltration process was that prepared using the following conditions in boxed ***Table*** 2.

Table 2 Membrane process condition and its properties

Coagulation temperature (the best 5 ° C) and Membarane Composition : dimethyl acetamide (81.57%) as solvent polyvinylpyrrolidone (1.68%) as additive and polysulfone (16.75 %) as membrane polymer filter.

This membrane gave :

Rejection coefficient of more than 90%
Flux as much as 20.6 L/m^2 hour.
Recommended operational temperature 5°C
and pressure for the enzyme concentration 2 kg/cm^2

for retaining maximum enzyme activity.

2.2. Ammonium sulfate precipitation

Isolation of the glucoamylase was carried out by addition of ammonium sulfate 80% saturation on the fermentation broth at 4°C (Udin et al, 1994).

Table 3. Isolation of the glucoamylase through 80% saturated ammonium sulfate fractionation.

Fraction	Volume (ml)	Activity (U/ml)	Total Activity (Unit)	Protein concntion (mg/ml)	Specific activity (U/mg protein)	Purity (Fold)	Yield (%)
Fermtation Broth	2563	4.40	11268	2.54	1.73	1.0	100
Residual dialysed 80% Ammonium sulfate	80	13.55	1084	4.18	3.24	1.87	9.6

The precipitate formed after centrifugation at 9000 rpm for 30 minutes was then dialysed in buffer solution (Table 3). It was found that the specific activity of the enzyme was around twice the crude enzyme and the yield was low indicating inappropriate separation condition despite the use of simple and practical conditions. The molecular weight was found to be 36,000 daltons as determined by SDS gel electrophoresis. The optimum pH with soluble starch as substrate was at pH 4.5 and its optimum temperature was 55° C while the Km value was 0.027%. Its initial velocity for various starch substrates Table 4) in decreasing preferences were in the order of soluble starch, sago starch, corn starch, arenga and tapioca starch.

Table 4. Initial velocity of the glucoamylase on various starch substrates

Substrate	Initial velocity expressed as Unit/mg protein
Soluble starch	6.94
Arengga starch	1.63
Corn starch	1.73
Sago starch	1.83
Tapioca starch	1.26

3. Immobilization of the glucoamylase

Immobilized enzyme can be defined as enzymes which physically confined or localized in a certain defined region of space with retention of their catalytic activities and which can be used repeatedly and continuously. Commercial agar usually can also be used as a matrix exchange on glucoamylase entrapment experiment (Udin et al, 1993). The result of this experiment showed that commercial agar can be used with proper concentration of 3%. The optimum temperature and pH of the immobilised glucoamylase were 55° C and pH 4.5 respectively. Km and Vmax of the enzyme when entrapped by agar matrix were $1x10^{-3}$ % and 41.94 ppm/minute , and by alginate matrix were $2.4x10^{-3}$ % and 21.3 ppm/minute, while the values for the native enzyme were $0.8x10^{-3}$% and 74.71 ppm/minute.The relative affinity toward various starches such as soluble, arenga, corn, sago, and tapioca starches of the immobilized enzyme with agar matrix is higher than the alginate one (Table 5). However, the affinity of both are lower than of the free enzyme.

Table 5. Relative affinity (%) of immobilized and free glucoamylase on various starch substrates

Starch Substrate	Enzyme form		
	Agar matrix	Alginate matrix	Free Enzyme
Soluble	61	49	100
Arenga	57	45	83
Corn	57	48	69
Sago	59	49	81
Tapioca	57	47	86

On the tenth days of storage in refrigerator (4-8°C) the activity of the agar entrapped immobilized enzyme became 76.16% whereas those entrapped in alginate had lost all the activity and the free enzyme had a.remaining of 55% activity.

4. Application of the glucoamylase

Enzymatic production of glucose syrup employs both alpha-amylase and glucoamylase. Alpha-amylase acts during liquefaction while glucoamylase in the saccharification process. In the present study the glucoamylase was obtained from Rhizopus oryzae fermentation for 5 days using 4-liter scale fermentor (Karossi et al, 1995). The influence of single stage and multiple stage additions of the glucoamylase on alpha amylase (of Wako - Japan) liquefied sago starch indicated no significant difference on the saccharification.

It was also found that the presence of 0.2 - 0.8 mM Li+, Na+, K+, Mg++, Ca++ and Ba++ salts enchanced the glucoamylase activity (Table 6) whereas at the level of 1mM they acted as inhibitors yielding 40 - 60 % inactivation of the enzyme. The results of HPLC analysis of the saccharification product showed that the glucoamylase hydrolysed 83.3% of sago starch yielding free glucose.

Table 6. Relative Activation (%) of the glucoamylase by alkali and earth alkali cations

Concentration (mM)	Relative activity (%)					
	Li+	Na+	K+	Mg++	Ca++	Ba++
None	100	100	100	100	100	100
0.2	112	143	137	130	123	124
0.4	119	149	139	136	143	132
0.8	122	151	144	137	152	141

CONCLUSIONS

The following conclusions may be drawn from the presented results.

1. The optimum process condition for production of the glucoamylase based on laboratory fermentor up to 10 L capacity has been established employing simple medium with Rhizopus oryzae as fermenting microorganism.
2. A specified polysulfone membrane used for enzyme concentration gave good rejection coefficient and reasonable flux.
3. Immobilized glucoamylase within comercial agar could be stored in refrigerator for 10 days retaining its activity better than the free enzyme.
4. For saccharification, the enzyme activity was increased most by addition of 0.8 mM calcium ion.

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